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ECOLOGY OF SOIL MICROORGANISMS -- RELATIONSHIP BETWEEN THE NUMBER OF MICROORGANISMS IN THE SOIL AND THEIR CHEMICAL ACTIVITY

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16. Abstract It is extremely difficult to determine the types and numbers of microorganisms which are actually engaged in a given metabolic activity in the soil, and it has been frequently stated, usually fallaciously, that there is a "contradiction between the bacteria count and the activity." An examination of specific instances reveals that this "contradiction" is often a superficial one, since past studies of soil microbiology have paid little attention to the qualitative contents of the microorganisms counted, to the soil environment itself, or to the physiological state of the microorganisms in it. Currently used measuring methods, such as the dilution plate method or counting methods using microscopes, are inadequate, and more study must be devoted to other somewhat more promising methods such as staining, ATP determination, use of fluorescent antibodies, and especially autoradiography.

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ECOLOGY OF SOIL MICROORGANISMS -- RELATIONSHIP BETWEEN THE NUMBER OF MICROORGANISMS IN THE SOIL AND THEIR CHEMICAL ACTIVITY

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In studying the ecology of microoganisms as well as that of the higher animals and plants, the measurement of the types and numbers of members of communities is, needless to say, necessary for analysis of the communities. In research into soil microorganisms as well, from the dawn of the science until today, a considerable portion of the research effort has constantly been devoted to studying the methods of measuring the types and numbers of communities of microorganisms. Nevertheless, the situation still remains unsatisfactory in various ways. /9*

Methods of identifying the types of all the microorganisms present in the soil and of accurately measuring the entire numbers of all of them present do not exist at the present time and probably will not come into existence for some time to come. In order to indentify the types of microorganisms, it is necessary for the microorganisms to be separated purely from the soil onto a culturing medium. However, the number of microorganisms which will grow on the culturing medium will amount to no more than 1/10-1/1000 of the number of microorganisms which are stained together with the soil and are counted under a microscope [1]. In spite of many efforts to improve them, the numerical values obtained by counting methods using culturing media have merely been increased by several times to 10 times at the very most. This is far from filling up the differences amounting to 100 or 1000 times. Perhaps because of such considerations, Imshenetskiy

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* Numbers in the margin indicate pagination in the foreign text.

holds that we are handling only 10% of the total microbial flora in the natural state [2]. Besides, even though we may have succeeded in purely separating them on a culturing medium, there still are living in the soil many microbes whose genera and species are difficult to identify. The difficulties of research into the microbial flora in the soil have been well expressed by Ushikoshi in his general treatment of the subject [3].

As a result of their life, the microorganisms in the soil engage in various types of so-called metabolic activities in the soil. Indeed, it would be possible to mention microorganisms having the capacity of performing certain metabolic activities after one had ascertained whether they had such metabolic capacities by means of pure strains. However, even if microorganisms having such metabolic capacities were present in the soil, it would not necessarily follow that the microorganisms were performing the metabolic activities in question in the soil. For example, with respect to Azotobacter[1], which fixes free nitrogen gas, it is at least possible to measure the number in the soil if a plate culture of a soil suspension is made on an agar plate containing a carbon source, but containing no nitrogen source. However, if the soil used for counting has been supplied with ammonium salts in the form of ammonium sulfate, it is

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[1] Attempts were made to inoculate live Azotobacter cells directly into the soil and to cause them to fix nitrogen for the purpose of utilizing it as nitrogen fertilizer. However, the amount of nitrogen which the Azotobacter would actually fix in the soil was less than 0.5 kg/N/ha per year, far less than the amount of nitrogen required by the crops (ordinary dry field crops are given about 10 kg N/ha as fertilizer, and horticultural crops are given several times this amount). Therefore, the plant growth-accelerating effects of Azotobacter inoculation which are sometimes seen have been denied in view of the nitrogen supply. The cause for the inoculation effects which are sometimes seen has been attributed to the fact that Azotobacter secretes antibodies against pathogenic microbes in plants and plant hormone-like substances [4].

probable that the Azotobacter which have been counted were utilizing the ammonium salts in their growth rather than fixing the free nitrogen as in the soil. As is seen in this one example, there are countless cases where the metabolic capacities of microorganisms vary depending upon the environmental conditions. It is precisely because of this universally known property that -- and I repeat -- it is difficult to find out the types and numbers of microorganisms which are actually engaged in a given metabolic activity in the soil. Here let us consider briefly some questions connected with this.

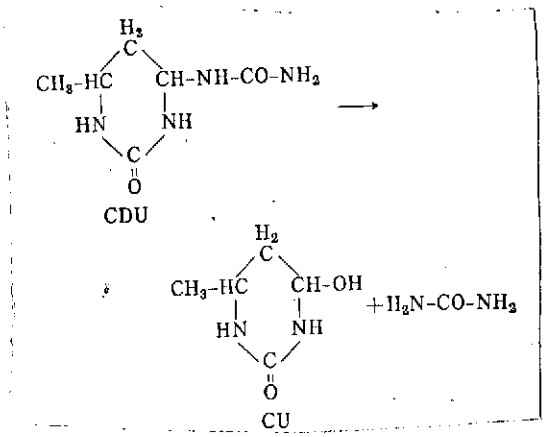
Contradictions between Bacteria Counts in the Soil and Their Activity

When one considers the question of how one is to find out the types and numbers of microorganisms actually engaged in metabolic activities in the soil, one must necessarily touch upon the question which has been grasped superficially under the expression "the contradiction between the bacteria count and their activity" in the history of soil microbiological research and which has been abandoned as a "contradiction". That is, let us suppose, on the one hand, that we measure the activity, such as the amount of oxygen absorbed in the soil, the amount of carbonic acid gas generated, and the amount of ammonia from organic matter which has been converted into inorganic forms. On the other hand, let us count the number of microorganisms in the soil. When we investigate the relationship between both sets of measured values, if the system is a simple one there ought to be an increase in the activity in direct proportion to the increase in the number of microorganisms. However, it has been the case only too often that no clear-cut proportional relationship at all has been observed in the soil between both sets of measured values. In the very few cases where a proportional relationship has been observed between both values, this has been regarded as entirely natural, while in the cases where

it has not been observed, it has been stated that there was a "contradiction between the bacteria count and the activity." Since no easy solution has been found for this "contradiction," the matter has been allowed to remain unsolved. This "contradiction" has not merely remained a simple phenomenological "contradiction;" it has, in fact, even divided soil microbiological research into two separate camps. That is, to borrow the expressions of Pramer [5], soil microbiologists have been divided up into those of the "S (soil) type," who study the metabolism of the soil, and those of the "M (microbes) type," who study the microbial flora. Even though both have dealt with the same topics, they have proceeded with their research independently of the other. This has given rise to distrust of methods of counting microorganisms in the soil, particularly since the dilution plate methods have been used to measure only very few of the microbes in the soil. The impasse reached by the M type research, which places chief emphasis on enumeration, has even led the S type persons to accuse their M type colleagues contemptuously of "enumeratomania" (numerare = number, mania = madness, "counting madness") [6]. /10

Such profound antagonism would no doubt never have come about had there been an explanation of the reason why this phenomenon of "contradiction between the bacteria count and the activity" comes about. However, as mentioned above, the "contradiction" could find no easy solution. Nevertheless, it was not that there were no studies delving into the reasons why the "contradiction" occurs. Here let us take up some of these studies.

2-oxo-4-methyl-6-ureido hexahydropyrimidine is a slow-acting nitrogen fertilizer[2] which is commonly sold on the market under the brand name CDU. When the urea radical in its side chain is removed by an acid hydrolysis reaction, 2-oxo-4-methyl-6-hydroxy hexahydropyrimidine (CU) is produced.



When the soil is irrigated with this CU, the number of bacteria will increase in direct proportion to the amount of CU added. However, as for the relationship between the rate of decomposition of CU in the soil and the amount of CU added, the results obtained appeared at first glance to be chaotic, as is shown in Fig. 1. Since the number of bacteria increased in direct proportion to the amount of CU added, as long as each of the cells of the CU decomposing bacteria in the irrigated soil were able to decompose CU at an equal speed, one would assume that the CU decomposition rate in the soil ought to have a simple proportional relationship to the amount of CU added. The fact that this was not the case may be called another phenomenon

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[2] Synthetic organic fertilizers which, rather than supplying nitrogen in inorganic form, as in ammonium sulfate or ammonium nitrate, supply it in organic form. The nitrogen is utilized by the plants after it has been converted into inorganic form in the soil by the action of microorganisms or by acid hydrolysis reactions. The object is to assure an effective period of fertilization longer than that of inorganic fertilizers.

illustrating the "contradiction between the bacteria count and the activity." The CU decomposition rate of the soil in Fig. 1 actually displayed two proportional relationships towards the amount of CU added. Furthermore, there were present two types of CU decomposing bacteria with different CU decomposing capacities. The predominant types of the CU decomposing bacteria were different in the soil displaying one proportional relationship and in the soil displaying the other proportional relationship. This was the reason why a phenomenon occurred which seemed at first glance to indicate a "contradiction between the bacteria count and the activity" [7]. These two types of bacteria differ in a number of their properties, but both belong to the Corynebacterium [8].

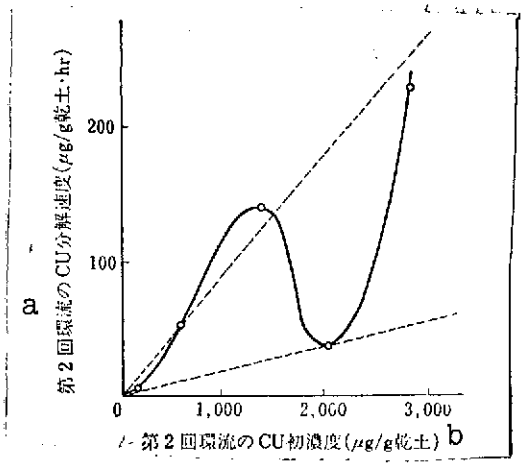

Fig. 1. Relationship between CU decomposition rate and initial CU concentration during second irradiation of soil irrigated with CU solution.

Key: a. CU decomposition rate during second irrigation (μg/g dry soil·hour); b. Initial CU concentration during second irrigation (μg/g dry soil)

This is a simple case of findings which appeared at first glance to involve a "contradiction," but which were really not "contradictory" at all when the types of the CU decomposing bacteria were investigated.

Watanabe et al. [9] measured the numbers of live bacteria (by the dilution plate method) and the oxygen absorption rate of the soil at different depths in fields plowed with a number of plowing methods. There were larger number of live bacteria nearer the surface layers and smaller numbers in the deeper layers.

However, the oxygen absorption rate of the soil did not decrease with the depth to the same degree as the decrease in the number of live bacteria. Large numbers of measured values were organized and plotted in Fig. 2. It is generally believed that the oxygen absorption rate ought to be proportional to the number of bacteria. If this were so, the ratio of the number of bacteria to the oxygen absorption rate, the B/R plotted on the ordinate of Fig. 2, ought to remain constant regardless of the number of bacteria on the abscissa. However, this was not so in their results. There was a smaller B/R ratio in the lower layers of the soil, where there were smaller numbers of live bacteria. In other words, there was a larger oxygen absorption rate per unit number of bacteria in the lower layers.

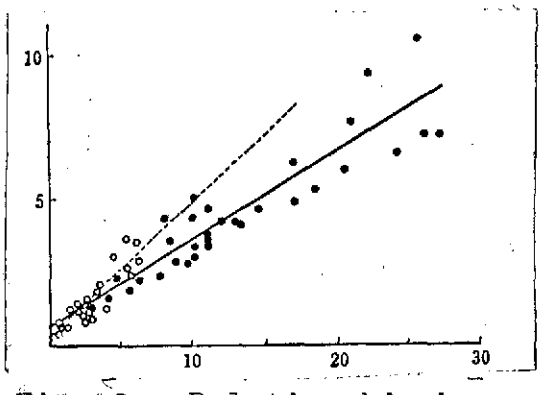

Fig. 2. Relationship between bacteria count/endogenous O_2 absorption ratio and bacteria count [9].
Y axis: bacteria count/endogenous O_2 absorption (10^6/µl/hour)
X axis: bacteria count (10^6/g dry soil)
● indicates measured value in plower layer,
o measured value in unplowed layer.

These measurements were made using a manometer to measure the oxygen absorption rate of the soil to which no substrates were added. However, it was said that there were no doubts about the procedures, especially when the lower layers of soil with smaller numbers of live bacteria were taken from the field and set in the /11
manometer for measurement. That is, the supplying of oxygen (which was lacking in the field state) which accompanied the procedure, the disturbance of the soil, and the measurement temperature (30°C), which was higher than the temperature in the field, contributed to enhancing the activity of the bacteria. The available organic matter, which had remained in the lower

layers of field soil unused by the bacteria, was rapidly used by the bacteria inside the manometer. As a result, the bacteria in the lower soil layers came to display a far higher oxygen absorption rate in the manomter than they had in the field. Accordingly, this was the reason why the oxygen absorption rate per unit number of bacteria was apparently larger in the lower layers of soil.

There is also the following discussion connected with this. Babiuk and Paul [10] counted the numbers of bacteria and ray fungi in unplowed soil under a microscope and then converted the counts into the weights of their organisms. The values throughout the year were 12-27 g (dry weight)/m^2 in soil depths of 0-10 cm and 32-76 g/m^2 in soil depths of 0-30 cm. However, the various types of organic substrates supplied to the soil by plants had values of about 500 g/m^2·year, and most of them were concentrated in the 0-10 cm layers. If calculations were made of the maintenance energy required for the renovation of the unstable cell components of microorganisms, it would amount to more than one-half of the available energy supplied to the soil, and the microorganisms would be able to multiply only several times. In other words, this means that most of the bacteria in the soil are normally in a physiologically inactive state and that those bacteria which are in the lower soil layers, where smaller amounts of substrates are supplied, are more poorly nourished. Consequently, the oxygen absorption rate per unit number of these bacteria in situ in the soil ought to be lower in the lower soil layers.

The use of a manometer to measure the oxygen absorption rate of the soil is a standard technique in soil microbiological research. However, the studies of Watanabe et al. indicate that there are cases when this measuring technique cannot be used to measure the in situ oxygen absorption rate of bacteria. This

example shows that it is difficult not only to find out the types and numbers of microorganisms which are actually engaged in soil metabolism, but even to measure the rate of *in situ* metabolism itself.

Let us mention another example of investigations of phenomena which displayed "contradiction between the bacteria count and the activity." Since the nitrification reaction of the soil is carried out exclusively by nitrifying bacteria which are autotrophic, such as *Nitrosomonas* or *Nitrobacter*, it is believed that the rate of the nitrification reaction, which is an energy acquisition reaction, is directly proportional to the number of nitrifying bacteria in the soil. However, Nishio and Furusaka [11] continued to irrigate the soil repeatedly while replacing the irrigating liquid with fresh nitrite solutions. When they measured the oxidation rate of the nitrous acid ions and the number of bacteria oxidized by nitrous acid, they found that there was only a slight increase in the oxidation rate of the nitrous acid ions as the irrigation was repeated. However, they observed the following phenomenon: that is, the number of bacteria oxidized by nitrous acid continued to increase to a remarkable degree (Fig. 3). When soil is irrigated repeatedly two or three times with nitrite solution, the oxidation rate of the nitrous acid ions in the soil will reach a more or less constant value and will then remain unchanged. For this reason, it was assumed in the past that the nitrification rate and the number of nitrifying bacteria were proportional to each other. Under this assumption, it had been concluded in the past, without measuring the numbers of bacteria, that the numbers of bacteria oxidized by nitrous acid in such soil would also reach a definite maximum (this soil is called "soil saturated with nitrifying bacteria") [12]. However, in their system, even though the nitrification rate reached a more or less constant value, the number of nitrifying bacteria continued to increase.

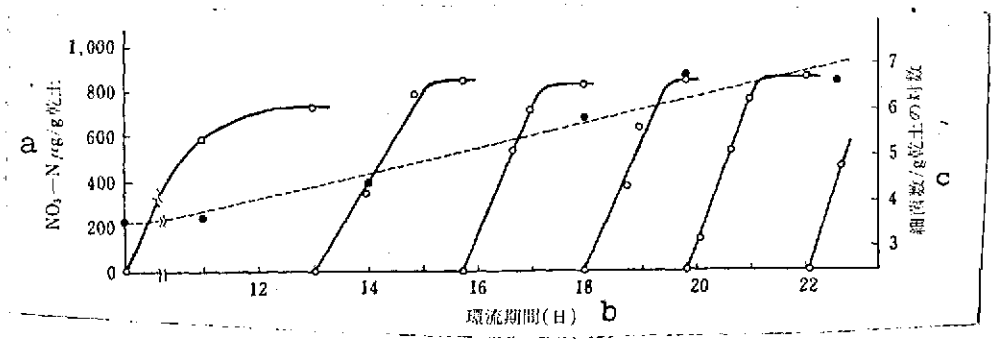

Fig. 3. Chronology of formation of nitric acid ions in soil irrigated with 10 mM sodium nitrite solution (-o-) and changes in number of bacteria oxidized by nitrous acid (-●-) [11].

Key: a. Dry soil
b. Period of irrigation (days)
c. Logarithm of bacteria count/g dry soil

Analyzing this phenomenon, they made the following observations. They collected samples of irrigated soils, sought the initial rates of the nitrification rates when nitrite of various concentrations was given while shaking it, and obtained the maximum nitrification rate from the Michaelis-Menten equation. When they compared the maximum nitrification rate in this dispersed soil with the nitrification rate in the irrigated state (approximately the same as the maximum nitrification rate in the irrigated state), they found that both were more or less identical in the initial period of irrigation, but that the difference between both became increasingly larger as irrigation was repeated. Thus, the maximum nitrification rate at the dispersed state gradually became higher than the rate in the irrigated state. Furthermore, when mold fungi were formed into pellets in a liquid culture, as the pellets became denser and larger, there also appeared differences in the supply of oxygen and substrate between the surfaces and the interiors of the pellets, and multiplication of the hyphae were confined to the surfaces of the pellets. As a result, the weight of the mycelium and the amounts of oxygen absorbed began to increase in cubic root fashion,

rather than in the normal logarithmic fashion, with respect to the culturing time [13, 14]. In exactly the same way, the oxidation of the nitric acid ions increased logarithmically with respect to the irrigation time during the initial period, but a cubic root increase was displayed in the later period. From these observations, they derived the conclusion illustrated in the model in Fig. 4 [15]. That is, during the initial period of irrigation, which is the stage where the number of bacteria is small, the bacteria are present on the surfaces of the soil particles either individually or in the form of small colonies. Therefore, each cell is able to obtain an adequate supply of substrate and oxygen, and there is logarithmic multiplication. However, as the bacteria come to multiply in lump form and the colonies gradually become larger, the supply of substrate and oxygen is now limited to the surface layers. The cells on the surface layers carry out nitrification reactions briskly, but those on the interior fall into a state of starvation and are unable to carry out nitrification reactions in the irrigated state. It is only when the soil is dispersed that they become able to oxidize the substrate. The rate of nitrification of irrigated soil is attributable to the cells which were active in the irrigated state. However, when the nitrifying bacteria are counted, the measurements are taken after culturing for nearly 1 month in a state where both the cells which were active in the irrigated soil and those which were in a state of starvation are mixed together. For this reason, even though the total

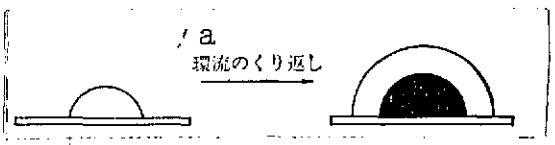

Fig. 4. Model diagram of mode of multiplication of nitrous acid-oxidized bacteria in soil irrigated with nitrite [15]. The cells on the surface of the cell lump (white) are active, but but the cells on the interior (black) are inactive.

Key: a. Repetition of irrigation

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number of bacteria continues to increase, as is shown in Fig. 3, there is only a very slight increase in the nitrification rate.

This example shows that it is necessary to recognize the mode in which the microorganisms are present in the soil and their physiological state.

The three studies mentioned above are among the very few studies which have traced down phenomena indicating "contradiction between the bacteria count and the activity" and which have made it clear why this "contradiction" exists. However, there have been observations in which the numbers of microorganisms in the soil and their chemical activity were directly proportional. Furusaka and Sato [16] as well as Nio and Furusaka [17] irrigated the soil with a glycine solution. They observed that the glycine oxidation rate and the number of bacteria were exactly proportional, as shown in Table 1, at the time when the glycine was oxidized and the bacteria multiplied rapidly. In the glycine-irrigated soil which they studied, bacteria having glycine oxidizing capacity multiplied rapidly and specifically until finally almost all of the bacteria in the soil were Pseudomonas putida, which has glycine oxidation capacity [18]. Furthermore, most of these bacteria were distributed around the outside of the pellets, where they could easily receive supplies of substrate. Since it was possible to count these bacteria in ordinary culture media, a perfect proportional relationship was obtained.

Furusaka and Sato [16] list three reasons why there occur phenomena indicating "contradictions between the bacteria count and the activity." (1) The species of microorganisms which is performing the activity in question is not counted selectively. (2) Even the same species of microorganisms may have different activity depending upon its physiological state. (3) The activity differs depending upon the microenvironmental conditions

TABLE 1. RELATIONSHIP BETWEEN GLYCINE OXIDATION RATE AND BACTERIA COUNT [16]

Time (hr)	M/150 glycine*			M/50 glycine*		
	Q_{O_2} $\mu l/g$ soil	Bacteria count x 10^6/g soil	Q_{O_2} $\mu l/10^8$ cells	Q_{O_2} $\mu l/g$ soil	Bacteria count x10^6/g soil	Q_{O_2} $\mu l/10^8$ cells
15	9.9	49	20.2	8.5	52	16.3
42	46.8	234	20.0	42.5	131	32.4
64	27.0	104	26.0	73.0	413	17.7
136	14.5	55	26.4	27.9	186	15.0

* Glycine concentration in irrigation liquid; soil weight is wet soil weight.

surrounding the microorganisms in the soil. Many studies in the past have shown "contradictions" between the bacteria count and the activity" because these points have been ignored and attempts have been made to make a mechanical connection between total counts of microorganisms, including different species of microorganisms and cells in different physiological states and definite metabolic rates.

Of the three conditions listed above, (2) and (3) are closely interconnected, and it is frequently difficult to distinguish between them. Of the studies mentioned above, the example of the soil irrigated with CU pertains to condition (1). The example of the relationship between the number of bacteria in the field and the oxygen absorption rate and the example of the soil irrigated with nitrite pertain to conditions (2) and (3). Viewed /13 from the reverse standpoint, the three conditions pointed out by Furusaka and Sato indicate that phenomena which indicate "contradictions between the bacteria count and the activity" are splendid phenomena for analyzing problems of soil microorganisms connected with these three conditions. That is, they reveal that in the past studies of soil microbiology, which have left the

"Contrad

"contradictions between the bacteria count and the activity" unsolved, not only was there little study of the qualitative contents of the microorganisms counted, but these was also very little importance attached to understanding the soil environment or the physiological state of the microorganisms in it. They also reveal that development of measuring methods taking these factors into consideration has lagged behind.

Concerning Measuring Methods

Next let us consider, although in general terms, the problems concerning measuring methods connected with what has been stated thus far. These problems concerning measuring methods may be divided up into a number of categories. The first problems are those of measuring the types and numbers of microorganisms actually engaged in a certain metabolic activity, taking their physiological states into consideration.

The first step here is to consider the question of differentiating in the count between the live cells in the soil and the dead cells, which naturally have no metabolic capacities. The dilution plate method is currently in universal use as the method of measuring the number of live microorganisms in the soil. However, this method handles only part of the microflora in the soil. On the other hand, in counting methods using microscopes, which are capable of handling the total number of microorganisms in the soil, the dead microorganisms are also included in the counts. In this connection, if staining of the microorganisms and the soil is performed with esterified fluorescent coloring matter (such as fluorescein diacetate or dibutyrate), it will be theoretically possible to differentiate in the count between the live microbes and the dead ones because, in the live microbes, the acetyl and butyl radicals in the side chain can be removed by the esterase, and the coloring matter can give out fluorescent light [10]. Separately from this method of observation, attempts

have also been made to measure the quantities of live microorganisms by estimating the amounts of compounds which are present only in the live microorganisms and absent in the other soil components. Attention was previously focused on the nucleic acids. However, even after the death of an organism, the nucleic acids are accumulated in the soil. ATP is considered to be more promising because it is hydrolyzed immediately after the death of a microbe. Personnel connected with NASA first attempted to estimate the amounts of ATP in the soil, utilizing a luciferin-luciferase system, for the purpose of discovering whether living organisms were present on other celestial bodies, or whether the biochemical preconditions for their existence were present [19]. Aside from the original purpose, studies were subsequently made of the application of ATP to soil microbiological research. It was found that when the number of live microorganisms in the soil counted by the dilution plate method was multiplied by the mean ATP contents in the microorganisms measured separately, the resulting value is usually one to two orders lower than the estimated value of the ATP. It is believed that the main reason for this difference is the presence of microorganisms other than the aerobic bacteria which were counted (especially those with large cells) and the presence of other living organisms [20].

The methods of measuring the number (quantity) of live microbes in the soil by means of staining or determining the ATP require further study. However, even in live microbes, there are differences in the physiological activities between the spores and the nutritive cells. Especially in mold fungi, large numbers of spores are formed all at once, and large number of the spores are stored up in the soil. For this reason, differentiation between both in the counts is more necessary in mold fungi than in bacteria. Most of the mold fungi detected by the dilution plate method are attributable to spores. For this reason, the mold fungi are stained together with the soil, or the mold fungi

growing on a slide glass buried in the soil are stained, and the lengths of the mold fungi are measured. However, identification of the microbes is impossible with this method. If one wishes to identify them, it is necessary to wash the soil [21] or roots [22] repeatedly with sterilized water in order to remove as many of the spores as possible (the hyphae cling to the soil or the root hairs and cannot be washed away easily). Then inoculation is performed on agar plates, and the types of mold fungi originating in the hyphae which grow on the plates are identified. In this case, it is impossible to measure the lengths of the hyphae when the identified microbes were present in the soil or on the roots. Even the development of these measuring methods alone has enriched research on mold fungi in the soil greatly in comparison to what it had been before. What was enriched was the M type research in the mold fungus flora mentioned above, but hardly anything was done concretely about the relationship between them and metabolism. Methods of identifying the mold fungi present in the form of mycelia in the soil or on the roots are able to make semi-determinations of their frequency of appearance. Thus, if the methods are applied to mold fungi which clearly have a definite metabolic capacity, it ought to be possible to relate them to metabolism. In addition, the method of using a fluorescent antibody to mark a species (or group) of microorganisms in the soil having a specific metabolic capacity and of tracking them by means of a fluorescence microscope will no doubt be useful in the same sense as the above. Hope is entertained about the fluorescent antibody method in the sense that it is able to trace specific microorganisms <u>in situ</u>. However, it cannot detect bacteria in the unicellular state in the soil, and for this reason the desired bacteria cannot be detected until agar has been inoculated with a soil suspension and a microcolony has been prepared. For soil bacteria with a low bacteria density, the fluoresence antibody method is at the stage of the dilution plate method, which has an extremely high selectivity [23].

At any rate, the methods described thus far are unable to establish directly the type and the extent of the metabolic activities being performed in the soil by the detected microorganisms. For these purposes, they are still at the rudimentary stage.

In this connection, the autoradiography method is considered as one possible means for attaining some progress in the current situation, even though the progress may be slight. Thus far, it has been applied to soil microbiological research only by a few persons and only within a limited range. First, let us describe the situation in outline. In 1958, Grossbard attempted to apply it to soil microbiological research [24]. She took autoradiograms of ^{60}Co and ^{137}Ce labeled mold fungus bodies buried in the earth with X-ray films placed on the surface of the soil at a distance from the soil layers where the fungus bodies were located. Insufficient study was given to the conditions, and the only results obtained were undifferentiated black dots. It was not even possible to distinguish between each of the fungus bodies. Thus, before using the method in the soil, she made a number of preliminary studies with pure strain on agar. Quite recently, she took autoradiograms of ^{14}C labeled plant remains /14
laid on the surface of the soil and observed the progress of analysis of the remains [25, 26]. At this time, she was able to observe the mold fungi which had incorporated ^{14}C in the later period of decomposition from the pictures of the surfaces of the ^{14}C labeled plant remains (although during the initial period of decomposition there is a high concentration, there is blackening of the whole and nothing can be seen). A cover glass coated with gelatin was then inserted for a definite period of time into the soil surrounding the plant remains, autoradiograms of this were taken by the stripping method, and the mold fungi which had incorporated ^{14}C were recognized clearly (since they were located at a distance from the remains). Either these mold fungi had

come from the remains, or they had obtained the ^{14}C by some other biological route. Thus, Grossbard's autoradiography still remains at the level of fragmentary observations of the ecology of soil microorganisms under restricted conditions.

On the other hand, Furusaka [27] applied autoradiography to the observation of the microdistribution of sulfate-reducing bacteria in water-flooded soil. Culturing was performed after giving $Na_2{}^{35}SO_4$ to water-flooded soil in a glass box. The soil was washed to remove the unreduced $^{35}SO_4^{--}$. Then the glass plates were removed, a polyethylene cloth and the X-ray film were laid on the surface of the soil, and the autoradiograms were taken. In this method, the $^{35}S^{--}$ produced by reduction by the sulfate-reducing bacteria is immediately deposited in the soil as $Fe^{35}S$ and is not removed by washing. The silver particles in the X-ray film are blackened in the form of tiny spots. Thanks to this, it was possible to trace the state of development of the microdistribution of the sulfate-reducing bacteria in the soil.

Waid et al. [29], though not working with soil, cultured fallen beech leaves for two hours in ^{14}C-glucose and then took took autoradiograms of the leaves. They distinguished between the biologically active hyphae of the mold fungi living on the surfaces of the leaves and the hyphae which were not active.

The current state of application of autoradiography in soil microbiological research is as outlined above. If we are to move a step forward and to apply autoradiography to the differential counting of cells actually engaged in a certain metabolic activity in the soil and cells which are not, the contact method used by Furusaka and his colleagues will be unsuitable. Either the dipping method or the stopping method used by Grossbard will be suitable. Although ^{14}C is satisfactory for large microorganisms such as mold fungi, if we are also to observe bacteria and ray fungi, autoradiograms using ^{3}H would appear to be suitable, as

Grossbard has pointed out (although she herself has not used them). Although not working with the soil, Brock and Brock [30] administered ^{3}H-thymidine *in situ* to bacteria called *Leucothrix mucor* living on the surface of *Polysiphonia lanosa*, a red alga, and observed them by the dipping method. They report that it was possible to distinguish between the cells which had incorporated ^{3}H-thymidine and were multiplying and those which had not. There are no doubt problems which still require study before autoradiography can be directly applied to differential counting in connection with the activity of microorganisms in the soil. However, even in the soil, as far as studies of metabolic capacities restricted to bacteria which are morphologically easily distinguishable or to specific groups of microorganisms are concerned, the autoradiography method is regarded as being promising, as long as it is applied in the proper manner, and attempts to study it are awaited.

In the foregoing, we have described in outline the current situation in methods of counting microorganisms in the soil taking their physiological states into consideration, as well as the methods which can be expected to be promising in the immediate future. However, the situation is still extremely unsatisfactory, and the day is awaited when better methods can be used in a routine manner.

The studies of Watanabe et al., which revealed that the oxygen absorption rates of microorganisms in soil samples as measured by manometers are not the *in situ* values, impress on one the urgency of the problem. They also raise a second problem: that of measurement of the *in situ* values of the quantities and rates of the metabolic processes occurring in the soil. Although this would appear to be simple at first glance, there are many basic obstacles, and the *in situ* values are difficult to measure in very many cases, as is clear, for example, from the problem of the

production of antibiotics in the soil. Thus, there are the problem of raising the sensitivity of methods of estimating the metabolites and the problem of increasing the low extraction ratio from the soil (especially from volcanic ash soil, which is widespread in Japan). Even though the use of isotopes has facilitated handling considerably, when attempts are made to measure the metabolites of certain microorganisms, they are sometimes suddenly metabolized by other microorganisms into a different form. Therefore, there arises the problem of grasping the intermediate reactions in a series of food chains.

There is also a third set of problems connected with finding out the differences in environmental conditions in each microposition surrounding the microorganisms in the soil and of finding out the physiological states of the microorganisms in these positions. At the present stage, attempts have just begun to approach this problems from the direction of studies in the microdistribution of microorganisms in the soil. The reader is referred to Hattori's general presentation for information on this point [31].

Conclusion

As an environment for microorganisms, the soil is highly diversified, both macroscopically and microscopically. Each microorganism is living in it under specific environmental conditions, and the physiological states of each microorganism will differ according to these conditions. Needless to say, prominent emphasis must be placed on this point in study of the ecology of soil microorganisms. The aforementioned "contradictions between bacteria count and activity" focus attention on the importance of this point, and better measuring methods are desired so that it can be elucidated concretely.

The above discussion has been based on this point of view. However, one would be too rash if, out of excessive emphasis on in situ measurement or measurement "under specific environmental conditions," one were to reject as worthless all research and measurement methods which cannot directly grasp the in situ ecology of microorganisms. The ideal is to be able to directly grasp the in situ ecology of microorganisms. However, since this is impossible at the present time, the effort must be made to devise better measuring methods and to accumulate efforts aiming at using the currently available measuring methods to approach closer and closer to the in situ ecology of microorganisms.

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